

1 BIOPOLYMER MARKER INDICATIVE OF DISEASE STATE HAVING
2 A MOLECULAR WEIGHT OF 2753 DALTONS

3
4 FIELD OF THE INVENTION

5 This invention relates to the field of characterizing the
6 existence of a disease state; particularly to the utilization
7 of mass spectroscopy to elucidate particular biopolymer markers
8 indicative of disease state, and most particularly to specific
9 biopolymer sequences having a unique relationship to at least
10 one particular disease state.

11
12 BACKGROUND OF THE INVENTION

13 Methods utilizing mass spectrometry for the analysis of a
14 target polypeptide have been taught wherein the polypeptide is
15 first solubilized in an appropriate solution or reagent system.
16 The type of solution or reagent system, e.g., comprising an
17 organic or inorganic solvent, will depend on the properties of
18 the polypeptide and the type of mass spectrometry performed and
19 are well known in the art (see, e.g., Vorm et al. (1994) Anal.
20 Chem. 66:3281 (for MALDI) and Valaskovic et al. (1995) Anal.
21 Chem. 67:3802 (for ESI). Mass spectrometry of peptides is
22 further disclosed, e.g., in WO 93/24834 by Chait et al.

23 In one prior art embodiment, the solvent is chosen so that

1 the risk that the molecules may be decomposed by the energy
2 introduced for the vaporization process is considerably
3 reduced, or even fully excluded. This can be achieved by
4 embedding the sample in a matrix, which can be an organic
5 compound, e.g., sugar, in particular pentose or hexose, but
6 also polysaccharides such as cellulose. These compounds are
7 decomposed thermolytically into CO₂ and H₂O so that no residues
8 are formed which might lead to chemical reactions. The matrix
9 can also be an inorganic compound, e.g., nitrate of ammonium
10 which is decomposed practically without leaving any residues.
11 Use of these and other solvents are further disclosed in U.S.
12 Pat. No. 5,062,935 by Schlag et al.

13 Prior art mass spectrometer formats for use in analyzing
14 the translation products include ionization (I) techniques,
15 including but not limited to matrix assisted laser desorption
16 (MALDI), continuous or pulsed electrospray (ESI) and related
17 methods (e.g., IONSPRAY or THERMOSPRAY), or massive cluster
18 impact (MCI); these ion sources can be matched with detection
19 formats including linear or non-linear reflection time-of-
20 flight (TOF), single or multiple quadropole, single or multiple
21 magnetic sector, Fourier Transform ion cyclotron resonance
22 (FTICR), ion trap, and combinations thereof (e.g., ion-
23 trap/time-of-flight). For ionization, numerous

1 matrix/wavelength combinations (MALDI) or solvent combinations
2 (ESI) can be employed. Subattomole levels of protein have been
3 detected, for example, using ESI (Valaskovic, G. A. et al.,
4 (1996) Science 273:1199-1202) or MALDI (Li, L. et al., (1996)
5 J. Am. Chem. Soc. 118:1662-1663) mass spectrometry.

6 ES mass spectrometry has been introduced by Fenn et al.
7 (J. Phys. Chem. 88, 4451-59 (1984); PCT Application No. WO
8 90/14148) and current applications are summarized in recent
9 review articles (R. D. Smith et al., Anal. Chem. 62, 882-89
10 (1990) and B. Ardrey, Electrospray Mass Spectrometry,
11 Spectroscopy Europe, 4, 10-18 (1992)). MALDI-TOF mass
12 spectrometry has been introduced by Hillenkamp et al. ("Matrix
13 Assisted UV-Laser Desorption/Ionization: A New Approach to Mass
14 Spectrometry of Large Biomolecules," Biological Mass
15 Spectrometry (Burlingame and McCloskey, editors), Elsevier
16 Science Publishers, Amsterdam, pp. 49-60, 1990). With ESI, the
17 determination of molecular weights in femtomole amounts of
18 sample is very accurate due to the presence of multiple ion
19 peaks which all could be used for the mass calculation.

20 The mass of the target polypeptide determined by mass
21 spectrometry is then compared to the mass of a reference
22 polypeptide of known identity. In one embodiment, the target
23 polypeptide is a polypeptide containing a number of repeated

1 amino acids directly correlated to the number of trinucleotide
2 repeats transcribed/translated from DNA; from its mass alone
3 the number of repeated trinucleotide repeats in the original
4 DNA which coded it, may be deduced.

5 U.S. Patent No. 6,020,208 utilizes a general category of
6 probe elements (i.e., sample presenting means) with Surfaces
7 Enhanced for Laser Desorption/Ionization (SELDI), within which
8 there are three (3) separate subcategories. The SELDI process
9 is directed toward a sample presenting means (i.e., probe
10 element surface) with surface-associated (or surface-bound)
11 molecules to promote the attachment (tethering or anchoring)
12 and subsequent detachment of tethered analyte molecules in a
13 light-dependent manner, wherein the said surface molecule(s)
14 are selected from the group consisting of photoactive
15 (photolabile) molecules that participate in the binding
16 (docking, tethering, or crosslinking) of the analyte molecules
17 to the sample presenting means (by covalent attachment
18 mechanisms or otherwise).

19 PCT/EP/04396 teaches a process for determining the status
20 of an organism by peptide measurement. The reference teaches
21 the measurement of peptides in a sample of the organism which
22 contains both high and low molecular weight peptides and acts
23 as an indicator of the organism's status. The reference

TO THE COURT

1 concentrates on the measurement of low molecular weight
2 peptides, i.e. below 30,000 Daltons, whose distribution serves
3 as a representative cross-section of defined controls.
4 Contrary to the methodology of the instant invention, the '396
5 patent strives to determine the status of a healthy organism,
6 i.e. a "normal" and then use this as a reference to
7 differentiate disease states. The present inventors do not
8 attempt to develop a reference "normal", but rather strive to
9 specify particular markers which are evidentiary of at least
10 one specific disease state, whereby the presence of said marker
11 serves as a positive indicator of disease. This leads to a
12 simple method of analysis which can easily be performed by an
13 untrained individual, since there is a positive correlation of
14 data. On the contrary, the '396 patent requires a complicated
15 analysis by a highly trained individual to determine disease
16 state versus the perception of non-disease or normal
17 physiology.

18 Richter et al, Journal of Chromatography B, 726(1999) 25-
19 35, refer to a database established from human hemofiltrate
20 comprised of a mass database and a sequence database. The goal
21 of Richter et al was to analyze the composition of the peptide
22 fraction in human blood. Using MALDI-TOF, over 20,000
23 molecular masses were detected representing an estimated 5,000

1 different peptides. The conclusion of the study was that the
2 hemofiltrate (HF) represented the peptide composition of
3 plasma. No correlation of peptides with relation to normal
4 and/or disease states is made.

5 As used herein, "analyte" refers to any atom and/or
6 molecule; including their complexes and fragment ions. In the
7 case of biological molecules/macromolecules or "biopolymers",
8 such analytes include but are not limited to: proteins,
9 peptides, DNA, RNA, carbohydrates, steroids, and lipids. Note
10 that most important biomolecules under investigation for their
11 involvement in the structure or regulation of life processes
12 are quite large (typically several thousand times larger than
13 H₂O.

14 As used herein, the term "molecular ions" refers to
15 molecules in the charged or ionized state, typically by the
16 addition or loss of one or more protons (H⁺).

17 As used herein, the term "molecular fragmentation" or
18 "fragment ions" refers to breakdown products of analyte
19 molecules caused, for example, during laser-induced desorption
20 (especially in the absence of added matrix).

21 As used herein, the term "solid phase" refers to the
22 condition of being in the solid state, for example, on the
23 probe element surface.

1 As used herein, "gas" or "vapor phase" refers to molecules
2 in the gaseous state (i.e., in vacuo for mass spectrometry).

3 As used herein, the term "analyte desorption/ionization"
4 refers to the transition of analytes from the solid phase to
5 the gas phase as ions. Note that the successful
6 desorption/ionization of large, intact molecular ions by laser
7 desorption is relatively recent (circa 1988)--the big
8 breakthrough was the chance discovery of an appropriate matrix
9 (nicotinic acid).

10 As used herein, the term "gas phase molecular ions" refers
11 to those ions that enter into the gas phase. Note that large
12 molecular mass ions such as proteins (typical mass=60,000 to
13 70,000 times the mass of a single proton) are typically not
14 volatile (i.e., they do not normally enter into the gas or
15 vapor phase). However, in the procedure of the present
16 invention, large molecular mass ions such as proteins do enter
17 the gas or vapor phase.

18 As used herein in the case of MALDI, the term "matrix"
19 refers to any one of several small, acidic, light absorbing
20 chemicals (e.g., nicotinic or sinapinic acid) that is mixed in
21 solution with the analyte in such a manner so that, upon drying
22 on the probe element, the crystalline matrix-embedded analyte
23 molecules are successfully desorbed (by laser irradiation) and

1 ionized from the solid phase (crystals) into the gaseous or
2 vapor phase and accelerated as intact molecular ions. For the
3 MALDI process to be successful, analyte is mixed with a freshly
4 prepared solution of the chemical matrix (e.g., 10,000:1
5 matrix:analyte) and placed on the inert probe element surface
6 to air dry just before the mass spectrometric analysis. The
7 large fold molar excess of matrix, present at concentrations
8 near saturation, facilitates crystal formation and entrapment
9 of analyte.

10 As used herein, "energy absorbing molecules (EAM)" refers
11 to any one of several small, light absorbing chemicals that,
12 when presented on the surface of a probe, facilitate the neat
13 desorption of molecules from the solid phase (i.e., surface)
14 into the gaseous or vapor phase for subsequent acceleration as
15 intact molecular ions. The term EAM is preferred, especially in
16 reference to SELDI. Note that analyte desorption by the SELDI
17 process is defined as a surface-dependent process (i.e., neat
18 analyte is placed on a surface composed of bound EAM). In
19 contrast, MALDI is presently thought to facilitate analyte
20 desorption by a volcanic eruption-type process that "throws"
21 the entire surface into the gas phase. Furthermore, note that
22 some EAM when used as free chemicals to embed analyte molecules
23 as described for the MALDI process will not work (i.e., they do

1 not promote molecular desorption, thus they are not suitable
2 matrix molecules).

3 As used herein, "probe element" or "sample presenting
4 device" refers to an element having the following properties:
5 it is inert (for example, typically stainless steel) and active
6 (probe elements with surfaces enhanced to contain EAM and/or
7 molecular capture devices).

8 As used herein, "MALDI" refers to Matrix-Assisted Laser
9 Desorption/Ionization.

10 As used herein, "TOF" stands for Time-of-Flight.

11 As used herein, "MS" refers to Mass Spectrometry.

12 As used herein "MALDI-TOF MS" refers to Matrix-assisted
13 laser desorption/ionization time-of-flight mass spectrometry.

14 As used herein, "ESI" is an abbreviation for Electrospray
15 ionization.

16 As used herein, "chemical bonds" is used simply as an
17 attempt to distinguish a rational, deliberate, and
18 knowledgeable manipulation of known classes of chemical
19 interactions from the poorly defined kind of general adherence
20 observed when one chemical substance (e.g., matrix) is placed
21 on another substance (e.g., an inert probe element surface).
22 Types of defined chemical bonds include electrostatic or ionic
23 (+/-) bonds (e.g., between a positively and negatively charged

1 groups on a protein surface), covalent bonds (very strong or
2 "permanent" bonds resulting from true electron sharing),
3 coordinate covalent bonds (e.g., between electron donor groups
4 in proteins and transition metal ions such as copper or iron),
5 and hydrophobic interactions (such as between two noncharged
6 groups).

7 As used herein, "electron donor groups" refers to the case
8 of biochemistry, where atoms in biomolecules (e.g, N, S, O)
9 "donate" or share electrons with electron poor groups (e.g., Cu
10 ions and other transition metal ions).

11 With the advent of mass spectroscopic methods such as
12 MALDI and SELDI, researchers have begun to utilize a tool that
13 holds the promise of uncovering countless biopolymers which
14 result from translation, transcription and post-translational
15 transcription of proteins from the entire genome.

16 Operating upon the principles of retentate chromatography,
17 SELDI MS involves the adsorption of proteins, based upon their
18 physico-chemical properties at a given pH and salt
19 concentration, followed by selectively desorbing proteins from
20 the surface by varying pH, salt, or organic solvent
21 concentration. After selective desorption, the proteins
22 retained on the SELDI surface, the "chip", can be analyzed
23 using the CIPHERGEN protein detection system, or an equivalent

1 thereof. Retentate chromatography is limited, however, by the
2 fact that if unfractionated body fluids, e.g. blood, blood
3 products, urine, saliva, and the like, along with tissue
4 samples, are applied to the adsorbent surfaces, the biopolymers
5 present in the greatest abundance will compete for all the
6 available binding sites and thereby prevent or preclude less
7 abundant biopolymers from interacting with them, thereby
8 reducing or eliminating the diversity of biopolymers which are
9 readily ascertainable.

10 If a process could be devised for maximizing the diversity
11 of biopolymers discernable from a sample, the ability of
12 researchers to accurately determine the relevance of such
13 biopolymers with relation to one or more disease states would
14 be immeasurably enhanced.

1 SUMMARY OF THE INVENTION

2 The instant invention is characterized by the use of a
3 combination of preparatory steps in conjunction with SELDI mass
4 spectroscopy and time-of-flight detection procedures to
5 maximize the diversity of biopolymers which are verifiable
6 within a particular sample. The cohort of biopolymers verified
7 within a sample is then viewed with reference to their ability
8 to evidence at least one particular disease state; thereby
9 enabling a diagnostician to gain the ability to characterize
10 either the presence or absence of said at least one disease
11 state relative to recognition of the presence and/or the
12 absence of said biopolymer.

13 Although all manner of biomarkers related to all disease
14 conditions are deemed to be within the purview of the instant
15 invention and methodology, particular significance was given to
16 those markers and diseases associated with the complement
17 system and Syndrome X and diseases related thereto.

18 The complement system is an important part of non-clonal
19 or innate immunity that collaborates with acquired immunity to
20 destroy invading pathogens and to facilitate the clearance of
21 immune complexes from the system. This system is the major
22 effector of the humoral branch of the immune system, consisting
23 of nearly 30 serum and membrane proteins. The proteins and

1 glycoproteins composing the complement system are synthesized
2 largely by liver hepatocytes. Activation of the complement
3 system involves a sequential enzyme cascade in which the
4 proenzyme product of one step becomes the enzyme catalyst of
5 the next step. Complement activation can occur via two
6 pathways: the classical and the alternative. The classical
7 pathway is commonly initiated by the formation of soluble
8 antigen-antibody complexes or by the binding of antibody to
9 antigen on a suitable target, such as a bacterial cell. The
10 alternative pathway is generally initiated by various cell-
11 surface constituents that are foreign to the host. Each
12 complement component is designated by numerals (C1-C9), by
13 letter symbols, or by trivial names. After a component is
14 activated, the peptide fragments are denoted by small letters.
15 The complement fragments interact with one another to form
16 functional complexes. Ultimately, foreign cells are destroyed
17 through the process of a membrane-attack complex mediated
18 lysis.

19 The C4 component of the complement system is involved in
20 the classical activation pathway. It is a glycoprotein
21 containing three polypeptide chains (α , β , and γ). C4 is a
22 substrate of component C1s and is activated when C1s hydrolyzes
23 a small fragment (C4a) from the amino terminus of the α chain,

1 exposing a binding site on the larger fragment (C4b).

2 The native C3 component consists of two polypeptide
3 chains, α and β . As a serum protein, C3 is involved in the
4 alternative pathway. Serum C3, which contains an unstable
5 thioester bond, is subject to slow spontaneous hydrolysis into
6 C3a and C3b. The C3f component is involved in the regulation
7 required of the complement system which confines the reaction
8 to designated targets. During the regulation process, C3b is
9 cleaved into two parts: C3bi and C3f. C3bi is a membrane-bound
10 intermediate wherein C3f is a free diffusible (soluble)
11 component.

12 Complement components have been implicated in the
13 pathogenesis of several disease conditions. C3 deficiencies
14 have the most severe clinical manifestations, such as recurrent
15 bacterial infections and immune-complex diseases, reflecting
16 the central role of C3. The rapid profusion of C3f moieties
17 and resultant "accidental" lysis of normal cells mediated
18 thereby gives rise to a host of auto-immune reactions. The
19 ability to understand and control these mechanisms, along with
20 their attendant consequences, will enable practitioners to
21 develop both diagnostic and therapeutic avenues by which to
22 thwart these maladies.

23 In the course of defining a plurality of disease specific

1 marker sequences, special significance was given to markers
2 which were evidentiary of a particular disease state or with
3 conditions associated with Syndrome-X. Syndrome-X is a
4 multifaceted syndrome, which occurs frequently in the general
5 population. A large segment of the adult population of
6 industrialized countries develops this metabolic syndrome,
7 produced by genetic, hormonal and lifestyle factors such as
8 obesity, physical inactivity and certain nutrient excesses.
9 This disease is characterized by the clustering of insulin
10 resistance and hyperinsulinemia, and is often associated with
11 dyslipidemia (atherogenic plasma lipid profile), essential
12 hypertension, abdominal (visceral) obesity, glucose intolerance
13 or noninsulin-dependent diabetes mellitus and an increased risk
14 of cardiovascular events. Abnormalities of blood coagulation
15 (higher plasminogen activator inhibitor type I and fibrinogen
16 levels), hyperuricemia and microalbuminuria have also been
17 found in metabolic syndrome-X.

18 The instant inventors view the Syndrome X continuum in its
19 cardiovascular light, while acknowledging its important
20 metabolic component. The first stage of Syndrome X consists of
21 insulin resistance, abnormal blood lipids (cholesterol and
22 triglycerides), obesity, and high blood pressure
23 (hypertension). Any one of these four first stage conditions

1 signals the start of Syndrome X.

2 Each first stage Syndrome X condition risks leading to
3 another. For example, increased insulin production is
4 associated with high blood fat levels, high blood pressure, and
5 obesity. Furthermore, the effects of the first stage conditions
6 are additive; an increase in the number of conditions causes an
7 increase in the risk of developing more serious diseases on the
8 Syndrome X continuum.

9 A patient who begins the Syndrome X continuum risks
10 spiraling into a maze of increasingly deadly diseases. The next
11 stages of the Syndrome X continuum lead to overt diabetes,
12 kidney failure, and heart failure, with the possibility of
13 stroke and heart attack at any time. Syndrome X is a dangerous
14 continuum, and preventative medicine is the best defense.
15 Diseases are currently most easily diagnosed in their later
16 stages, but controlling them at a late stage is extremely
17 difficult. Disease prevention is much more effective at an
18 earlier stage.

19 Subsequent to the isolation of particular disease state
20 marker sequences as taught by the instant invention, the
21 promulgation of various forms of risk-assessment tests are
22 contemplated which will allow physicians to identify
23 asymptomatic patients before they suffer an irreversible event

1 such as diabetes, kidney failure, and heart failure, and enable
2 effective disease management and preventative medicine.
3 Additionally, the specific diagnostic tests which evolve from
4 this methodology provide a tool for rapidly and accurately
5 diagnosing acute Syndrome X events such as heart attack and
6 stroke, and facilitate treatment.

7 Accordingly, it is an objective of the instant invention
8 to define a disease specific marker sequence which is useful in
9 evidencing and categorizing at least one particular disease
10 state.

11 It is another objective of the instant invention to
12 evaluate samples containing a plurality of biopolymers for the
13 presence of disease specific marker sequences which evidence a
14 link to at least one specific disease state.

15 It is a further objective of the instant invention to
16 elucidate essentially all biopolymeric moieties contained
17 therein, whereby particularly significant moieties may be
18 identified.

19 It is a further objective of the instant invention provide
20 at least one purified antibody which is specific to said
21 disease specific marker sequence.

22 It is yet another objective of the instant invention to
23 teach a monoclonal antibody which is specific to said disease

1 specific marker sequence.

2 It is a still further objective of the invention to teach
3 polyclonal antibodies raised against said disease specific
4 marker.

5 It is yet an additional objective of the instant invention
6 to teach a diagnostic kit for determining the presence of said
7 disease specific marker.

8 It is a still further objective of the instant invention
9 to teach methods for characterizing disease state based upon
10 the identification of said disease specific marker.

11 Other objectives and advantages of this invention will
12 become apparent from the following description taken in
13 conjunction with the accompanying drawings wherein are set
14 forth, by way of illustration and example, certain embodiments
15 of this invention. The drawings constitute a part of this
16 specification and include exemplary embodiments of the present
17 invention and illustrate various objects and features thereof.

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19

20

1 BRIEF DESCRIPTION OF THE FIGURES

2 Figure 1 is a representation of derived data which
3 characterizes a disease specific marker having a particular
4 sequence useful in evidencing and categorizing at least one
5 particular state;

6 Figure 2 is the characteristic profile derived via
7 SELDI/TOF MS of the disease specific marker of Figure 1.

1 DETAILED DESCRIPTION OF THE INVENTION

2 Serum samples from individuals were analyzed using Surface
3 Enhanced Laser Desorption Ionization (SELDI) using the
4 CIPHERGEN PROTEINCHIP system. The chip surfaces included, but
5 were not limited to IMAC-3-Ni, SAX2 surface chemistries, gold
6 chips, and the like.

7 Preparatory to the conduction of the SELDI MS procedure,
8 various preparatory steps were carried out in order to maximize
9 the diversity of discernible moieties educable from the sample.
10 Utilizing a type of micro-chromatographic column called a C18-
11 ZIPTIP available from the Millipore company, the following
12 preparatory steps were conducted.

- 13 1. Dilute sera in sample buffer;
- 14 2. Aspirate and dispense ZIP TIP in 50% Acetonitrile;
- 15 3. Aspirate and dispense ZIP TIP in Equilibration;
16 solution;
- 17 4. Aspirate and Dispense in serum sample;
- 18 5. Aspirate and Dispense ZIP TIP in Wash solution;
- 19 6. Aspirate and Dispense ZIP TIP in Elution Solution.

20 Illustrative of the various buffering compositions useful
21 in the present invention are:

22 Sample Buffers (various low pH's): Hydrochloric acid
23 (HCl), Formic acid, Trifluoroacetic acid (TFA),

1 Equilibration Buffers (various low pH's): HCl, Formic
2 acid, TFA;

3 Wash Buffers (various low pH's): HCl, Formic acid, TFA;

4 Elution Solutions (various low pH's and % Solvents): HCl,
5 Formic acid, TFA;

6 Solvents: Ethanol, Methanol, Acetonitrile.

7 Spotting was then performed, for example upon a Gold Chip in
8 the following manner:

- 9 1. spot 2 ul of sample onto each spot
- 10 2. let sample partially dry
- 11 3. spot 1 ul of matrix, and let air dry.

12 **HiQ Anion Exchange Mini Column Protocol**

- 13 1. Dilute sera in sample/running buffer;
- 14 2. Add HiQ resin to column and remove any air bubbles;
- 15 3. Add Ul water to aid in column packing;
- 16 4. Add sample/running buffer to equilibrate column;
- 17 5. Add diluted sera;
- 18 6. Collect all the flow through fraction in Eppendorf
19 tubes until level is at resin;
- 20 7. Add sample/running buffer to wash column;
- 21 8. Add elution buffer and collect elution in Eppendorf
22 tubes.

23 Illustrative of the various buffering compositions useful

1 in this technique are:

2 Sample/Running buffers: including but not limited to
3 Bicine buffers of various molarities, pH's, NaCl content, Bis-
4 Tris buffers of various molarities, pH's, NaCl content,
5 Diethanolamine of various molarities, pH's, NaCl content,
6 Diethylamine of various molarities, pH's, NaCl content,
7 Imidazole of various molarities, pH's, NaCl content, Tricine of
8 various molarities, pH's, NaCl content, Triethanolamine of
9 various molarities, pH's, NaCl content, Tris of various
10 molarities, pH's, NaCl content.

11 Elution Buffer: Acetic acid of various molarities, pH's,
12 NaCl content, Citric acid of various molarities, pH's, NaCl
13 content, HEPES of various molarities, pH's, NaCl content, MES
14 of various molarities, pH's, NaCl content, MOPS of various
15 molarities, pH's, NaCl content, PIPES of various molarities,
16 pH's, NaCl content, Lactic acid of various molarities, pH's,
17 NaCl content, Phosphate of various molarities, pH's, NaCl
18 content, Tricine of various molarities, pH's, NaCl content.

19 **Chelating Sepharose Mini Column**

- 20 1. Dilute Sera in Sample/Running buffer;
- 21 2. Add Chelating Sepharose slurry to column and allow
22 column to pack;
- 23 3. Add UF water to the column to aid in packing;

- 1 4. Add Charging Buffer once water is at the level of the
2 resin surface;
- 3 5. Add UF water to wash through non bound metal ions
4 once charge buffer washes through;
- 5 6. Add running buffer to equilibrate column for sample
6 loading;
- 7 7. Add diluted serum sample;
- 8 8. Add running buffer to wash unbound protein;
- 9 9. Add elution buffer and collect elution fractions for
10 analysis;
- 11 10. Acidify each elution fraction.

12 Illustrative of the various buffering compositions useful
13 in this technique are: Sample/Running buffers including but not
14 limited to Sodium Phosphate buffers at various molarities and
15 pH's;

16 Charging buffers including but not limited to Nickel
17 Chloride, Nickel Sulphate, Copper II Chloride, Zinc Chloride or
18 any suitable metal ion solution;

19 Elution Buffers including but not limited to Sodium
20 phosphate buffers at various molarities and pH's containing
21 various molarities of EDTA and/or Imidazole.

22
23

1 **HiS Cation Exchange Mini Column Protocol**

- 2 1. Dilute sera in sample/running buffer;
- 3 2. Add HiS resin to column and remove any air bubbles;
- 4 3. Add Uf water to aid in column packing;
- 5 4. Add sample/running buffer to equilibrate column for
- 6 sample loading;
- 7 5. Add diluted sera to column;
- 8 6. Collect all flow through fractions in Eppendorf tubes
- 9 until level is at resin.
- 10 7. Add sample/running buffer to wash column.
- 11 8. Add elusion buffer and collect elusion in Eppendorf
- 12 tubes.

13 Illustrative of the various buffering compositions

14 useful in this technique are:

15 Sample/Running buffers: including but not limited to

16 Bicine buffers of various molarities, pH's, NaCl content,

17 Bis-Tris buffers of various molarities, pH's, NaCl content,

18 Diethanolamine of various molarities, pH's, NaCl content,

19 Diethylamine of various molarities, pH's, NaCl content,

20 Imidazole of various molarities, pH's, NaCl content, Tricine

21 of various molarities, pH's, NaCl content, Triethanolamine of

22 various molarities, pH's, NaCl content, Tris of various

23 molarities, pH's, NaCl content.

1 Elution Buffer: Acetic acid of various molarities,
2 pH's, NaCl content, Citric acid of various molarities, pH's,
3 NaCl content, HEPES of various molarities, pH's, NaCl
4 content, MES of various molarities, pH's, NaCl content, MOPS
5 of various molarities, pH's, NaCl content, PIPES of various
6 molarities, pH's, NaCl content, Lactic acid of various
7 molarities, pH's, NaCl content, Phosphate of various
8 molarities, pH's, NaCl content, Tricine of various
9 molarities, pH's, NaCl content.

10 The procedure for profiling serum samples is described
11 below:

12 Following the preparatory steps illustrated above,
13 various methods for use of the PROTEINCHIP arrays, available
14 for purchase from CIPHERGEN Biosystems (Palo Alto, CA), may
15 be practiced. Illustrative of one such method is as follows.

16 The first step involved treatment of each spot with 20
17 ml of a solution of 0.5 M EDTA for 5 minutes at room
18 temperature in order to remove any contaminating divalent
19 metal ions from the surface. This was followed by rinsing
20 under a stream of ultra-filtered, deionized water to remove
21 the EDTA. The rinsed surfaces were treated with 20 ml of 100
22 mM Nickel sulfate solution for 5 minutes at room temperature

1 after which the surface was rinsed under a stream of ultra-
2 filtered, deionized water and allowed to air dry.

3 Serum samples (2 ml) were applied to each spot (now
4 "charged" with the metal-Nickel) and the PROTEINCHIP was
5 returned to the plastic container in which it was supplied.
6 A piece of moist KIMWIPE was placed at the bottom of the
7 container to generate a humid atmosphere. The cap on the
8 plastic tube was replaced and the chip allowed to incubate at
9 room temperature for one hour. At the end of the incubation
10 period, the chip was removed from the humid container and
11 washed under a stream of ultra-filtered, deionized water and
12 allowed to air dry. The chip surfaces (spots) were now
13 treated with an energy-absorbing molecule that helps in the
14 ionization of the proteins adhering to the spots for analysis
15 by Mass Spectrometry. The energy-absorbing molecule in this
16 case was sinapinic acid and a saturated solution prepared in
17 50% acetonitrile and 0.05% TFA was applied (1 ml) to each
18 spot. The solution was allowed to air dry and the chip
19 analyzed immediately using MS (SELDI).

20 Serum samples from patients suffering from a variety of
21 disease states were analyzed using one or more protein chip
22 surfaces, e.g. a gold chip or an IMAC nickel chip surface as
23 described above and the profiles were analyzed to discern

1 notable sequences which were deemed in some way evidentiary
2 of at least one disease state.

3 In order to purify the disease specific marker and
4 further characterize the sequence thereof, additional
5 processing was performed.

6 For example, Serum (20 ml) was (diluted 5-fold with
7 phosphate buffered saline) concentrated by centrifugation
8 through a YM3 MICROCON spin filter (Amicon) for 20 min at
9 10,000 RPM at 4°C in a Beckman MICROCENTRIFuge R model bench
10 top centrifuge. The filtrate was discarded and the retained
11 solution, which contained the two peptides of interest, was
12 analyzed further by tandem mass spectrometry to deduce their
13 amino acid sequences. Tandem mass spectrometry was performed
14 at the University of Manitoba's (Winnipeg, Manitoba, Canada)
15 mass spectrometry laboratory using the procedures that are
16 well known to practitioners of the art.

17 As a result of these procedures, the disease specific
18 marker DAHKSEVAHFRFKDLGEENFKALVL was found. This marker is
19 characterized as a Serum Albumin having a molecular weight of
20 about 2753 daltons. The characteristic profile of the marker
21 is set forth in Figure 2. As easily deduced from the data
22 set forth in Figure 1, this marker is indicative of insulin
23 resistance.

1 In accordance with various stated objectives of the
2 invention, the skilled artisan, in possession of the specific
3 disease specific marker as instantly disclosed, would readily
4 carry out known techniques in order to raise purified
5 biochemical materials, e.g. monoclonal and/or polyclonal
6 antibodies, which are useful in the production of methods and
7 devices useful as point-of-care rapid assay diagnostic or
8 risk assessment devices as are known in the art.

9 The specific disease markers which are analyzed
10 according to the method of the invention are released into
11 the circulation and may be present in the blood or in any
12 blood product, for example plasma, serum, cytolyzed blood,
13 e.g. by treatment with hypotonic buffer or detergents and
14 dilutions and preparations thereof, and other body fluids,
15 e.g. CSF, saliva, urine, lymph, and the like. The presence
16 of each marker is determined using antibodies specific for
17 each of the markers and detecting specific binding of each
18 antibody to its respective marker. Any suitable direct or
19 indirect assay method may be used to determine the level of
20 each of the specific markers measured according to the
21 invention. The assays may be competitive assays, sandwich
22 assays, and the label may be selected from the group of well-
23 known labels such as radioimmunoassay, fluorescent or

1 chemiluminescence immunoassay, or immunoPCR technology.
2 Extensive discussion of the known immunoassay techniques is
3 not required here since these are known to those of skilled
4 in the art. See Takahashi et al. (Clin Chem 1999;45(8):1307)
5 for S100B assay.

6 A monoclonal antibody specific against the disease
7 marker sequence isolated by the present invention may be
8 produced, for example, by the polyethylene glycol (PEG)
9 mediated cell fusion method, in a manner well-known in the
10 art.

11 Traditionally, monoclonal antibodies have been made
12 according to fundamental principles laid down by Kohler and
13 Milstein. Mice are immunized with antigens, with or without,
14 adjuvants. The splenocytes are harvested from the spleen for
15 fusion with immortalized hybridoma partners. These are
16 seeded into microtitre plates where they can secrete
17 antibodies into the supernatant that is used for cell
18 culture. To select from the hybridomas that have been plated
19 for the ones that produce antibodies of interest the
20 hybridoma supernatants are usually tested for antibody
21 binding to antigens in an ELISA (enzyme linked immunosorbent
22 assay) assay. The idea is that the wells that contain the
23 hybridoma of interest will contain antibodies that will bind

1 most avidly to the test antigen, usually the immunizing
2 antigen. These wells are then subcloned in limiting dilution
3 fashion to produce monoclonal hybridomas. The selection for
4 the clones of interest is repeated using an ELISA assay to
5 test for antibody binding. Therefore, the principle that has
6 been propagated is that in the production of monoclonal
7 antibodies the hybridomas that produce the most avidly
8 binding antibodies are the ones that are selected from among
9 all the hybridomas that were initially produced. That is to
10 say, the preferred antibody is the one with highest affinity
11 for the antigen of interest.

12 There have been many modifications of this procedure
13 such as using whole cells for immunization. In this method,
14 instead of using purified antigens, entire cells are used for
15 immunization. Another modification is the use of cellular
16 ELISA for screening. In this method instead of using
17 purified antigens as the target in the ELISA, fixed cells are
18 used. In addition to ELISA tests, complement mediated
19 cytotoxicity assays have also been used in the screening
20 process. However, antibody-binding assays were used in
21 conjunction with cytotoxicity tests. Thus, despite many
22 modifications, the process of producing monoclonal antibodies

1 relies on antibody binding to the test antigen as an
2 endpoint.

3 The purified monoclonal antibody is utilized for
4 immunochemical studies.

5 Polyclonal antibody production and purification
6 utilizing one or more animal hosts in a manner well-known in
7 the art can be performed by a skilled artisan.

8 Another objective of the present invention is to provide
9 reagents for use in diagnostic assays for the detection of
10 the particularly isolated disease specific marker sequences
11 of the present invention.

12 In one mode of this embodiment, the marker sequences of
13 the present invention may be used as antigens in immunoassays
14 for the detection of those individuals suffering from the
15 disease known to be evidenced by said marker sequence. Such
16 assays may include but are not limited to: radioimmunoassay,
17 enzyme-linked immunosorbent assay (ELISA), "sandwich" assays,
18 precipitin reactions, gel diffusion immunodiffusion assay,
19 agglutination assay, fluorescent immunoassays, protein A or G
20 immunoassays and immunoelectrophoresis assays.

21 According to the present invention, monoclonal or
22 polyclonal antibodies produced against the disease specific
23 marker sequence of the instant invention are useful in an

1 immunoassay on samples of blood or blood products such as
2 serum, plasma or the like, spinal fluid or other body fluid,
3 e.g. saliva, urine, lymph, and the like, to diagnose patients
4 with the characteristic disease state linked to said marker
5 sequence. The antibodies can be used in any type of
6 immunoassay. This includes both the two-site sandwich assay
7 and the single site immunoassay of the non-competitive type,
8 as well as in traditional competitive binding assays.

9 Particularly preferred, for ease and simplicity of
10 detection, and its quantitative nature, is the sandwich or
11 double antibody assay of which a number of variations exist,
12 all of which are contemplated by the present invention. For
13 example, in a typical sandwich assay, unlabeled antibody is
14 immobilized on a solid phase, e.g. microtiter plate, and the
15 sample to be tested is added. After a certain period of
16 incubation to allow formation of an antibody-antigen complex,
17 a second antibody, labeled with a reporter molecule capable
18 of inducing a detectable signal, is added and incubation is
19 continued to allow sufficient time for binding with the
20 antigen at a different site, resulting with a formation of a
21 complex of antibody-antigen-labeled antibody. The presence
22 of the antigen is determined by observation of a signal which

1 may be quantitated by comparison with control samples
2 containing known amounts of antigen.

3 All patents and publications mentioned in this
4 specification are indicative of the levels of those skilled
5 in the art to which the invention pertains. All patents and
6 publications are herein incorporated by reference to the same
7 extent as if each individual publication was specifically and
8 individually indicated to be incorporated by reference.

9 It is to be understood that while a certain form of the
10 invention is illustrated, it is not to be limited to the
11 specific form or arrangement herein described and shown. It
12 will be apparent to those skilled in the art that various
13 changes may be made without departing from the scope of the
14 invention and the invention is not to be considered limited
15 to what is shown and described in the specification and
16 drawings/figures.

17 One skilled in the art will readily appreciate that the
18 present invention is well adapted to carry out the objectives
19 and obtain the ends and advantages mentioned, as well as
20 those inherent therein. The oligonucleotides, peptides,
21 polypeptides, biologically related compounds, methods,
22 procedures and techniques described herein are presently
23 representative of the preferred embodiments, are intended to

1 be exemplary and are not intended as limitations on the
2 scope. Changes therein and other uses will occur to those
3 skilled in the art which are encompassed within the spirit of
4 the invention and are defined by the scope of the appended
5 claims. Although the invention has been described in
6 connection with specific preferred embodiments, it should be
7 understood that the invention as claimed should not be unduly
8 limited to such specific embodiments. Indeed, various
9 modifications of the described modes for carrying out the
10 invention which are obvious to those skilled in the art are
11 intended to be within the scope of the following claims.

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